*THE CHEMICAL NATURE OF THE RNA-AMINO ACID COMPOUND FORMED BY AMINO ACID-ACTIVATING ENZYMES**

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Specific enzymes which catalyze the formation of amino acyl adenylates have been isolated from bacterial, plant, and animal sources.[1–5] More recently it was found that these enzymes also catalyze the formation of an amino acyl-RNA compound, and the evidence from a number of laboratories[6–10] favors the mechanism shown in equations (1) to (3).

$$\text{ATP} + \text{amino acid} \rightleftharpoons \text{amino acyl-AMP} + \text{PP} \tag{1}$$

$$\text{Amino acyl-AMP} + \text{RNA} \rightleftharpoons \text{amino acyl-RNA} + \text{AMP} \tag{2}$$

$$\text{ATP} + \text{amino acid} + \text{RNA} \rightleftharpoons \text{amino acyl-RNA} + \text{AMP} + \text{PP}. \tag{3}$$

The acceptor RNA, which represents about 10 per cent of the cellular RNA of *Escherichia coli*, is of relatively low molecular weight.[11] Each amino acid is linked to a specific site on the RNA and these acceptor sites function independently of one another.[6–8]

Recently, Hecht *et al.*[12] showed that the acceptor activity of liver RNA required a specific terminal sequence of nucleotides, i.e., adenylyl 5′-3′-cytidylyl-5′-3′-cytidylyl-5′-3′-RNA (RNApCpCpA[13]). It seemed likely that this grouping provides either the point of attachment of amino acids to the RNA or that this terminal sequence is required for the binding of the RNA to the activating enzyme. The experiments described in the present report were designed to distinguish between these two possibilities.

Our results show that the leucine-, valine-, and methionine-activating enzymes link the respective amino acids to the RNA through the 2′- or 3′-hydroxyl group of the ribose of the terminal nucleotide bearing the unesterified 3′-hydroxyl group. We have identified the terminal nucleotide of the chain which accepts leucine as adenylic acid by isolating leucyl-2′- or 3′-adenosine from RNase digests of leucyl-RNA. From the behavior of the bound amino acids to treatment with hydroxylamine,[6, 7, 14] we have inferred that the linkage is an acyl ester. Based on the present

concept of the structure of RNA, i.e., that there is no branching of the polynucleotide chain, we have concluded that there is only one amino acid bound per polynucleotide chain. These findings, together with the observations of Zachau *et al.*[14] that a terminal adenylic acid unit is also the acceptor for leucine in liver "soluble" RNA, support the idea that the adenylic acid in the terminal sequence adenylyl 5′-3′-cytidylyl 5′-3′-cytidylyl 5′-3′-RNA may be the acceptor for all amino acids[12] and that the remainder of the RNA chain functions in determining which amino acid is linked to which chain.

General Methods and Materials.—Amino acid-activating enzymes specific for L-valine, L-methionine, and L-leucine were each purified on the basis of the ATP-PP^{32} exchange reaction,[2] from extracts of *E. coli* by methods to be reported later. With the purified enzymes, the rate of ATP-PP^{32} exchange with any other amino acid was less than 5 per cent of that observed with the specific amino acid.[15] Snake venom phosphodiesterase, free of phosphomonoesterase activity, was prepared by the method of Koerner and Sinsheimer;[16] semenphosphomonoesterase, free of phosphodiesterase activity, was obtained from Dr. L. A. Heppel of the National Institutes of Health; and crystalline pancreatic RNase was purchased from Worthington Biochemicals Corp. The amino acid-acceptor RNA was isolated from dried cells of *E. coli* by a procedure to be published later.

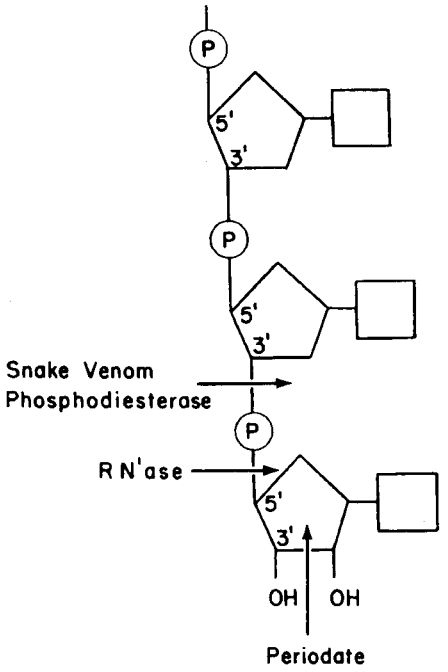


FIG. 1.—Action of periodate, snake venom phosphodiesterase, and RNase on a portion of a typical polynucleotide chain.

The quantity of amino acid linked to RNA was determined by incubating the appropriate C^{14}-labeled amino acid with an excess of the specific amino acid-activating enzyme, ATP, Mg^{++}, and RNA, and measuring the amount of radioactivity precipitated by 0.35 *N* perchloric acid, or by an ethanol-salt mixture (67 per cent ethanol–0.5 *M* NaCl). Under these conditions the amount of amino acid precipitated was directly proportional to the amount of RNA added[7] and we have used this value as a measure of the total number of specific binding sites for a given amino acid present in the RNA. On several occasions a mixture of C^{14}-labeled amino acids together with an unfractionated extract of *E. coli* was used to assay the RNA for total amino acid-acceptor activity.[7] Further details are presented with the individual experiments. Inorganic phosphate was measured by the method of Fiske and Subbarow.[17]

Experimental Results.—Requirement for specific terminal nucleotide unit for linking amino acids to RNA: Removal from the polynucleotide chain of the terminal unit bearing the unesterified 3′-hydroxyl group can be accomplished by oxidative cleavage of the ribose moiety with periodate (see Fig. 1), followed by alkaline

decomposition of the oxidized unit and enzymatic removal of the resulting phosphomonoester groups.[18] This procedure not only removes the original terminal nucleotide, but regenerates a new end having a free 3′-hydroxyl group. Treatment of the isolated acceptor-RNA from *E. coli* in this manner results in essentially complete loss of acceptor activity (Table 1). Periodate treatment alone (line 2) is also sufficient to inactivate the RNA, showing that the RNA must have an intact as well as a specific terminal nucleotide unit in order to accept amino acids.

Snake venom phosphodiesterase can degrade deoxy- and ribo-oligonucleotides, one nucleotide at a time, starting from the end bearing the free 3′-hydroxyl groups on the pentose.[19–22] Moreover, the enzyme attacks available ends at random rather than degrading one complete chain at a time.[21, 22] Phosphodiesterase digestion of amino acid-acceptor RNA results in essentially complete loss of acceptor activity when approximately 5 per cent of the nucleotides have been split off (Fig. 2). By contrast, spleen phosphodiesterase which degrades oligodeoxynucleotides from the nucleotide end bearing the free 5′-hydroxyl group,[21] but whose action on polyribonucleotides is not yet established, produced only 20 per cent loss of acceptor activity for an equivalent amount of degradation.

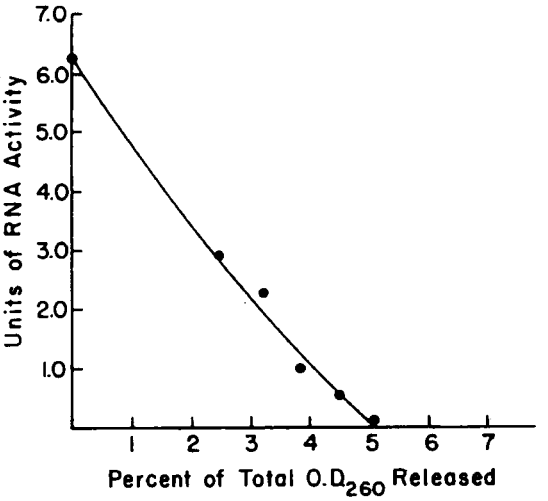


FIG. 2.—Degradation of the amino acid-acceptor RNA by snake venom phosphodiesterase.

Snake venom diesterase digestion: The final volume of 0.14 ml contained 0.14 *M* tris buffer, pH 8.7, 0.007 *M* $MgCl_2$, 0.5 mg of RNA, and 6.3 units of snake venom phosphodiesterase. The mixture was incubated at 37° and samples were removed at 0, 5, 10, 20, 30, and 60 min and heated for 2 min at 100°. The amount of degradation was determined by measuring the optical density at 260 mμ of the supernatant fluid following the addition of perchloric acid to the heated aliquot. Another portion of each sample was assayed for its ability to accept amino acids, using the mixed C^{14}-amino acids and unfractionated *E. coli* extract as previously described. The ordinate is expressed as units of RNA activity and 1 unit corresponds to the incorporation of 10^3 cpm under the standard conditions. Incubation of the RNA in the absence of the phosphodiesterase led to no loss in activity.

These two experiments show that a specific nucleotide unit at the end of the RNA chain having a free 3′-hydroxyl group is essential for the activity of the acceptor-RNA fraction from *E. coli*.

The terminal nucleotide as site of attachment for amino acids: If either of the hydroxyl groups of the ribose on the terminal nucleotide is the site of attachment for an amino acid, then treatment of the amino acid-RNA compound with periodate should not result in loss of acceptor activity. Specifically, this hypothesis states that if the leucyl-RNA is exposed to periodate, all sites other than the one specific for leucine should be inactivated and the leucine site should be protected. Table 2 describes an experiment in which leucyl-, valyl-, and methionyl-RNA were each treated with periodate, recovered by precipitation, and freed of the bound amino acids. The RNA was then tested for the ability to accept each of these amino

TABLE 1

EFFECT OF PERIODATE OXIDATION ON AMINO ACID-ACCEPTOR ACTIVITY OF *E. coli* RNA*

RNA Sample	Amount of Amino Acid Linked to RNA, mμ mole per μmole of Nucleotide	
	Leucine	Valine
1. Original RNA	0.67	0.39
2. Periodate-treated RNA	0.00	0.02
3. Two incubated at pH 10	..	0.02
4. Three treated with phosphomonoesterase	0.00	0.02
5. Original RNA incubated at pH 10	..	0.33
6. Original RNA treated with phosphomonoesterase	0.69	0.37

The periodate treatment of the RNA was carried out as follows: 30 mg of RNA in 0.1 *M* succinate buffer, pH 5.6, was incubated for 30 min at 20° with 2 to 5 μmoles of sodium metaperiodate. This represents a 2- to 5-fold excess of periodate over the calculated number of end groups assuming that there are on the average 100 nucleotides per RNA chain.[11] The RNA was recovered by precipitation with alcohol, dialyzed against water, and then used as such, or the solution was made 0.1 *M* with glycine buffer, pH 10.3, and incubated at 20° for 16 hr. The alkali-treated RNA was recovered by precipitation with the ethanol-salt mixture. Phosphatase treatment was carried out on the alkali-treated RNA as follows: approximately 20 mg of the RNA was incubated at 37° with 22 units of purified semen phosphomonoesterase (1 unit = 1 μmole of phosphate removed from adenosine 3′-phosphate per hour) at pH 5.0 in the presence of 0.01 *M* Mg^{++}. Estimation of the amount of inorganic phosphate liberated showed that 0.61 μmole of phosphate was split off. This corresponded to 94 per cent of the amount expected on the basis of the assumed chain length of 100 nucleotides. The polynucleotide minus the original terminal nucleotide was reisolated by precipitation with ethanol and salt and assayed as described below.

The amount of amino acid linked to RNA was determined as follows: The reaction mixture volume was 0.5 ml and contained 0.1 *M* sodium cacodylate buffer, pH 7.0, 0.002 *M* $MgCl_2$, 0.01 *M* ATP, 0.2 mg of bovine serum albumin, 0.002 *M* glutathione, 0.0003 *M* C^{14}-DL-leucine or C^{14}-DL-valine (specific activity 3.5 to 6.0 × 10^6 cpm per μmole) and either 0.9 μg of the purified leucine-activating enzyme or 0.5 μg of the valine-activating enzyme. Incubation was for 20 min at 30°, at which time the incorporation was complete. The RNA was precipitated with ethanol-salt, washed, and counted in a windowless-gas-flow counter with appropriate self-absorption corrections. Testing of control mixtures of inactivated acceptor-RNA and active RNA under these conditions showed no decrease in the yield of RNA-amino acid formed.

* This loss of amino acid-acceptor activity occurs for all amino acids since the incorporation of a mixture of C^{14}-labeled amino acids in the presence of a crude extract of *E. coli* containing many activating enzymes[7] is likewise prevented by treatment with periodate.

TABLE 2

ASSAY OF RNA ISOLATED AFTER TREATMENT OF AMINO ACID-RNA COMPOUNDS WITH PERIODATE

Amino Acid Tested	Original RNA		Leucyl-RNA		Valyl-RNA		Methionyl-RNA	
	Periodate Treatment							
	+	−	+	−	+	−	+	−
	Amino Acid Incorporation, mμmole per μmole of Nucleotide							
Leucine	0.01	0.59	0.56	0.54	<0.01	0.54	<0.01	..
Valine	0.04	0.30	0.04	0.28	0.23	0.30	0.03	..
Methionine	<0.005	0.13	<0.005	0.12	<0.005	0.13	0.15	0.15

The different C^{14}-amino acid-RNA compounds were prepared by scaling up the usual assay reaction mixtures described in Table 1. Five to 10 mg of the amino acid-RNA compound were incubated with periodate as described previously and then reisolated by alcohol precipitation and dialysis. Approximately 85 to 90 per cent of the optical density units and radioactivity was recovered. The C^{14}-amino acids were removed by treatment with 0.1 *M* glycine buffer, pH 10.3 at 30° for 60 min and the amino acid-free RNA was recovered by precipitation. The yield of RNA was between 80 and 90 per cent of the initial input. The different RNA preparations were then assayed for their ability to accept leucine, valine, and methionine by the methods described previously. In the case of the methionine assay, 0.00025 *M* C^{14}-L-methionine (specific activity 4.9 × 10^6 cpm per μmole) and 30 μg of a purified L-methionine activating-enzyme from *E. coli* were used.

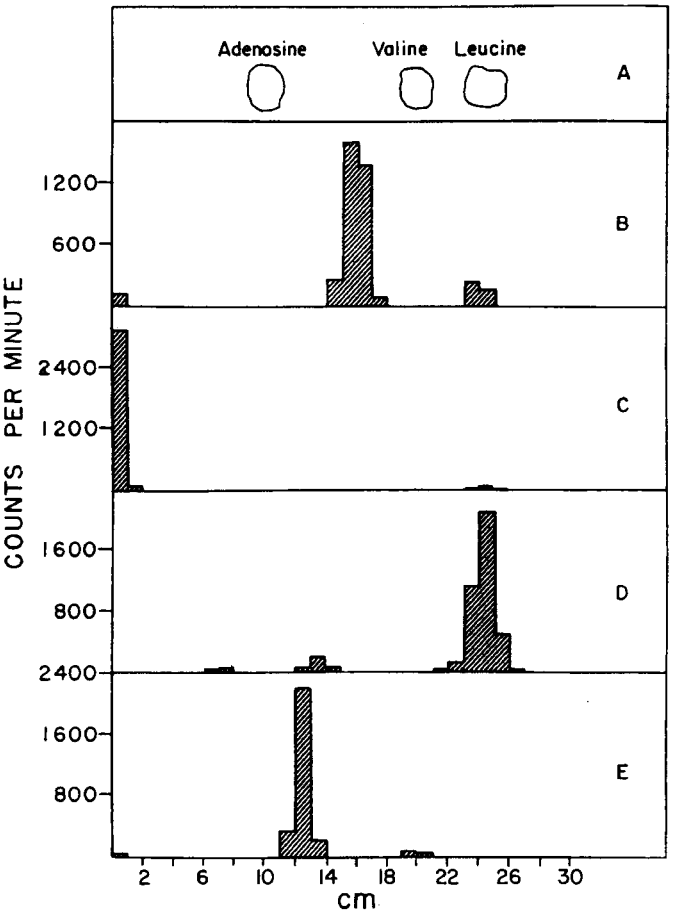


FIG. 3.—Analysis of chromatograms of RNase digests of C^{14}-leucyl and C^{14}-valyl RNA.

(*A*) Adenosine, valine, and leucine markers. (*B*) C^{14}-leucyl-RNA + RNase. (*C*) C^{14}-leucyl-RNA. (*D*) C^{14}-leucyl-RNA treated with 0.01 *N* KOH. (*E*) C^{14}-valyl-RNA + RNase.

The samples used for chromatography were prepared as follows: Approximately 0.5 mg of C^{14}-leucyl- or valyl-RNA, prepared as described in Table 1, were incubated with 0.3 μg of crystalline RNase at pH 5.8, for 15 min at 30°, and then an aliquot was placed on Whatman No. 1 filter paper strips and chromatographed at 3° with sec-butanol:formic acid:H_2O (70:10:20).[25] Another sample of leucyl-RNA was incubated in 0.01 *N* KOH for 5 min at 20°. This procedure was insufficient to cause any breakdown of the RNA. Following chromatography, the individual strips were cut into 1-cm sections, eluted with 0.05 *N* NH_4OH, and the eluates counted. The amount of radioactivity per 1-cm strip is plotted against the distance along the paper.

Not shown here are chromatograms of C^{14}-valyl-RNA, and C^{14}-valyl-RNA treated with alkali. Valyl-RNA remained at the starting point, while in the latter experiment all the radioactivity migrated the same distance as free valine.

acids. The results show that these amino acids protect only their own sites on the RNA against inactivation by periodate. Based on current ideas of polynucleotide structure, the only periodate-sensitive groups are the terminal nucleotides bearing unesterified 3′-hydroxyl groups. Assuming that the polynucleotide chain is un-

branched, we can conclude that such a chain has a single amino acid-binding site and that this is the 2′- or 3′-hydroxyl group of the terminal nucleotide having the free 3′-hydroxyl group. Moreover, since there are specific sites for each different amino acid, there must be a different polynucleotide chain for each amino acid.

Identification of the terminal nucleotide to which leucine is linked: Pancreatic ribonuclease (RNase) digestion of RNA yields nucleosides from those end groups which have a free 3′-hydroxyl group and which are adjacent to a pyrimidine nucleo-

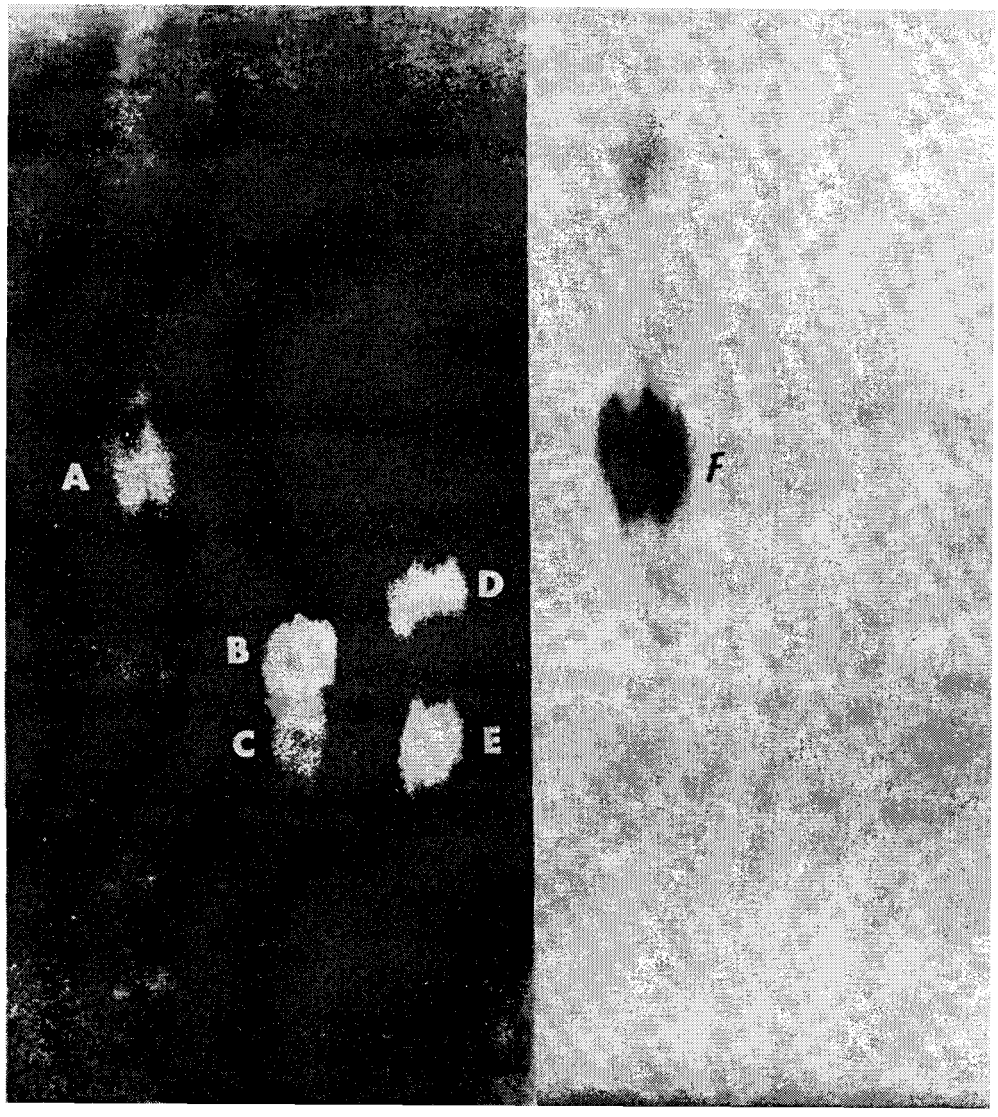
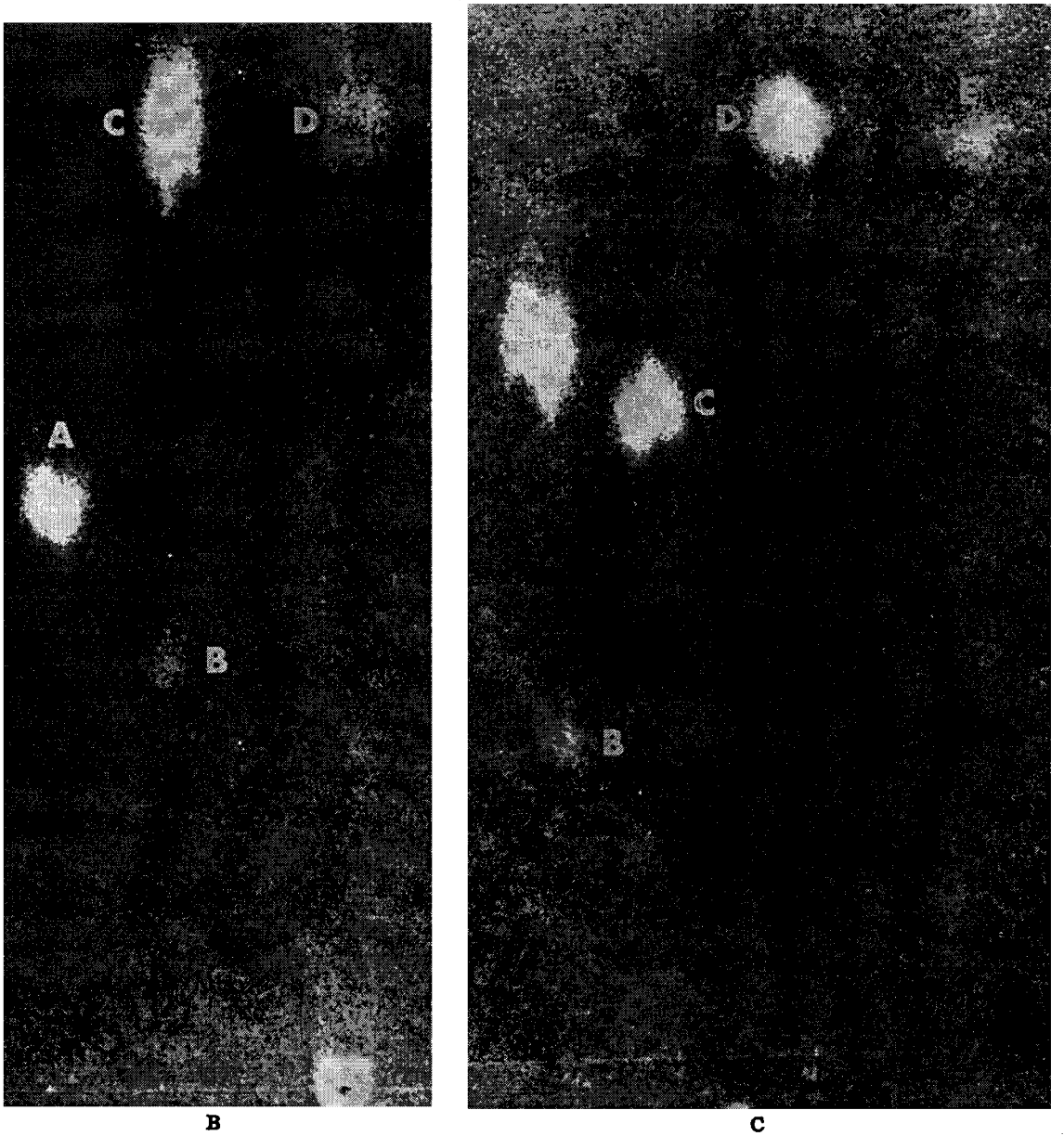


FIG. 4.—Chromatographic identification of leucyl-adenosine as product produced by RNase digestion of leucyl-RNA.

(*a*) Chromatogram and radioautogram of the isolated C^{14}-leucyl-adenosine and ribonucleoside markers in the solvent described in Figure 3. The various spots are (*A*) C^{14}-leucyl-adenosine, (*B*) adenosine, (*C*) cytidine, (*D*) uridine, and (*E*) guanosine. At the right (*F*) is the X-ray overlay showing the position of the radioactivity. The small amount of radioactivity at the top of the radioautogram represents free leucine derived from breakdown of the leucyl-adenosine during the chromatographic run.

tide.[24] It was reported earlier[7] that amino acids linked to RNA were converted from an acid-insoluble form to an acid-soluble form. Chromatography of the acid-soluble fraction from RNase digests of C^{14}-leucyl- and C^{14}-valyl-RNA showed that all of the amino acids migrated to a region slightly ahead of that occupied by the free nucleosides and behind the free amino acids (Figs. 3, *A*, 3, *B*, and 4, *A*). The



B C

(*b*) Chromatogram of the alkaline hydrolysate of the C^{14}-leucyl-adenosine in *n*-butanol:isobutyric acid:NH_4OH.[26] The leucyl-adenosine was exposed to 0.01 *N* KOH for 5 min at 30°. The various spots are: (*A*) uridine, (*B*) guanosine, (*C*) adenosine, and (*D*) hydrolysate.

(*c*) Chromatogram of the acid-hydrolysate of the isolated adenosine in *n*-butanol:isobutyric acid:NH_4OH.[26] The adenosine was hydrolyzed in 1 *N* HCl for 15 min at 100°. The various spots are: (*A*) adenosine, (*B*) guanosine, (*C*) uracil, (*D*) adenine, and (*E*) hydrolysate.

leucine-bound component was isolated by successive paper chromatographic runs (Fig. 4, *A*) and subjected to dilute alkaline hydrolysis. All of the radioactivity now migrated with free leucine and the ultraviolet-absorbing material chromatographed as adenosine (Fig. 4, *B*). The isolated adenosine, when hydrolyzed with acid, gave quantitative recovery of an ultraviolet-absorbing material which was identified as adenine (Fig. 4, *C*). We infer that the leucine was linked to one of the two *cis*-hydroxyl groups of the ribose since the R_f of the leucyl-adenosine was unaffected by treatment with periodate while free adenosine, subjected to the same procedure, gave a spot with an altered R_f (0.37 as compared to 0.30 in the butanol-formic acid solvent). We have concluded from this experiment that the terminal nucleotide which binds leucine is, as was found by Zachau *et al.*[14] with liver "soluble" RNA, adenylic acid, and, moreover, from the known reactivity of the bound amino acids to hydroxylamine[6, 7, 14] that the linkage is from the amino acid carboxyl group to the 2′- or 3′-hydroxyl group of the ribose.

Discussion.—Amino acid-activating enzymes catalyze a two-step reaction by which amino acids become linked to specific sites of RNA. The present results, together with those of Hecht *et al.*[12] and Zachau *et al.*[13] show that these sites are terminal nucleotides bearing a free 3′-hydroxyl group on the ribose, and that the attachment of the amino acid to the RNA is very likely by an ester linkage. If, as suggested by Hecht *et al.*,[12] all amino acid-acceptor ends are terminated by an identical sequence of at least three nucleotides (RNApCpCpA), then either the structure or the composition and sequence of nucleotides in the remaining portion of the chain must determine which amino acid is linked to which chain. It seems likely that this specificity results from the interaction of a portion of the polynucleotide chain with specific amino acid-activating enzymes. Consistent with such a hypothesis are preliminary studies which show that the polynucleotide, from which the terminal nucleotide has been removed by the successive action of periodate, alkali, and phosphomonoesterase, competitively inhibits the rate of amino acid incorporation into active RNA, but does not affect the yield. The periodate oxidation technique we have used has enabled us to make preparations which are specific for single amino acids but which still contain the inactive chains. It is clear that the answer to the specificity problem will require the isolation of homogeneous preparations of polynucleotide chains specific for a single amino acid.

It may be of some interest to point out one difference between the properties of the isolated mammalian and the bacterial amino acid-acceptor RNA. Zachau *et al.*[14] and Hoagland[27] have reported that the isolated "soluble" RNA from liver is already saturated with respect to many amino acids and the incorporation of labeled amino acids represents an exchange process. This is not the case with the acceptor-RNA as we have isolated it from *E. coli*, for if amino acids were already linked to the RNA it would not have been possible to completely inactivate the original RNA with periodate. This difference between the mammalian and bacterial RNA preparations may be simply the result of the different procedures used to isolate the RNA rather than some fundamental difference in the two types of RNA.

Zachau *et al.*[13] have pointed out that this type of acyl ester linkage, i.e., the amino acid-ribonucleotide compound, represents a new type of "high-energy bond." Studies on the measurement of the equilibrium constant for the enzymatic reaction

linking valine to RNA indicate a value of 0.32 at pH 7.0 at 30°,[28] showing that only a small free energy change is involved in forming an amino acyl-RNA compound from ATP, the free amino acid, and RNA.

Summary.—Purified leucine-, valine-, and methionine-activating enzymes from *E. coli* catalyze the transfer of these amino acids to a specific fraction of RNA according to the following general equation:

$$\text{ATP} + \text{amino acid} + \text{RNA} \rightleftharpoons \text{amino acid-RNA} + \text{PP} + \text{AMP}.$$

The acceptor-activity of the RNA is lost after destruction or removal of the terminal nucleotide having the free 3′-hydroxyl group by periodate or the action of snake venom phosphodiesterase. If an amino acid is linked to the RNA prior to treatment with periodate, the acceptor site specific for the bound amino acid is protected against inactivation while all others are destroyed. Treatment of leucyl-RNA with pancreatic RNase results in the liberation of leucyl 2′- or 3′-adenosine. All of these observations are consistent with the hypothesis that each amino acid is linked to a specific polynucleotide chain through linkage of the amino acid to the 2′- or 3′-hydroxyl group of the terminal nucleotide of the chain.

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† American Cancer Society Postdoctoral Research Fellow.

‡ National Science Foundation Predoctoral Research Fellow.

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